(51) International Patent Classification 5 : C07K	A2	(11) International Publication Number: WO 93/24517
		(43) International Publication Date: 9 December 1993 (09.12.93)

(54) Title: PEPTIDE SUB-LIBRARY KITS

(57) Abstract

The subjects of the invention are kits comprising free or support bound peptide mixtures (sub-libraries) as components, methods for their preparation and method for their use in discovery of bioactive peptides as potential drugs. Groups of free peptide mixtures (sub-libraries) forming components of the kit may carry different color or fluorescent labels facilitating their identification. The solid support in kits comprising support bound peptides may carry color or fluorescent labels as well, but the size or the specific gravity of the beads are also used as markers. The kits can be applied in all kinds of biological screening tests, including binding tests with macromolecules (like proteins), cells or tissues. The amino acid sequences of bioactive peptides can generally be determined in three stages: (i) determination of amino acid occurrence list by use of amino acid tester sub-kit, (ii) determination of occurrence library by use of the first sub-kit, and (iii) determination of sequences by use of second sub-kits.

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Peptide sub-library kits.

The subject of the invention comprises special peptide kits, processes for their synthesis and methods for their application in identification of biologically active peptides (named "domino strategy") which can be utilized as potential drugs. Components of the kits are multicomponent mixtures of free or support bound peptides or peptide derivatives. Submission in logical order of components of the kits to binding experiments with living cells, tissues or proteins or execution of other kinds of biological or biochemical tests, and evaluation of their result, makes possible to determine the amino acid sequence of the bioactive peptides.

The invention is based on the highly efficient synthetic method introduced by the inventors and their colleagues: (i) Furka et al. Abstr. 14th Int. Congr. Biochem., Prague, Czechoslovakia, 1988, Vol 5, p. 47.; (ii) Furka et al. Abstr. 10th Int. Symp. Med.Chem., Budapest, Hungary, 1988, p. 288.; (iii) Furka et al. Int. J. Peptide Protein Res., 1991, 37, 487-493. This "portioning-mixing" method makes possible to prepare in a couple of days multicomponent peptide mixtures comprising, for example, all possible sequences of: tripeptides (8 thousand), tetrapeptides (160 thousand), pentapeptides (3.2 million) etc. It is a characteristic feature of this solid phase method that all peptide molecules formed on one bead of the solid support have the same sequence.

Later on Lam et al. (Nature, 1991, 354, 82-84) published a method identical with that of ours for the synthesis of support bound peptide mixtures and developed an identification procedure based on specific binding of protein molecules to the bead carrying the bioactive peptide, and determination of the sequence of the active peptide by using a sequenator. Houghten and his colleagues (Nature, 1991, 354, 85-86) described a slightly modified version of our synthetic method together with an iterative identification procedure applicable for mixtures of free peptides.

Fodor et al. (Science, 1991, 251, 767-773) also developed a new synthetic and identification method in which the peptides were synthesized in different but predetermined locations on the surface of a glass

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microscope slide. In the method of Scott and Smith (Science,1990, 249, 386-390) the peptide sequences are expressed on the surface of phage after inserting into its genome random nucleotide sequences.

Definitions and notations. The multicomponent peptide mixtures are usually called *peptide libraries*. Libraries synthesized by the "portioning-mixing" method can be deduced by varying amino acids in the different positions of peptides. This is experimentally accomplished by repeating the following operations: (i) dividing the support before coupling into equal portions, (ii) coupling a different amino acid to each portion, then (iii) mixing the portions. Before describing the invention in details some additional definitions are introduced.

The name *normal library* is used for mixtures formed by varying the same number of amino acids in all positions. If this is not the case, one can speak about *general libraries*. *Standard libraries* will represent a special case of the normal libraries where the 20 common amino acids are varied in all positions. Cysteine is often omitted and only the remaining 19 amino acids are varied. These mixtures are named *standard-C libraries*. A special combined library, containing a genetic series of standard libraries will be named *genetic library*. E.g., the genetic pentapeptide library includes all standard libraries from dipeptides to pentapeptides. The use of D-amino acids, derivatives of amino acids or non-common amino acids besides the common ones can be indicated by the name: *extended library*.

Any kind of library - except genetic libraries - may be considered as parent library and can be divided into sub-libraries. *Sub-libraries* are special partial libraries of parent libraries and can be synthesized by omitting the variation of the amino acids in one or more positions, while in the rest of positions they are varied like in the parent library. A non-varied position is occupied by the same amino acid in all peptides. Sub-libraries may be classified according their order: the first, second, third etc. order sub-libraries have one, two, three, etc. non-varied positions, respectively. Table 1 shows how the amino acids are varied in a first order standard pentapeptide sub-library.

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Table 1.

c	Varied amino acids
1	A,C,D,E,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y
2	E
3	A,C,D,E,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y
4	A,C,D,E,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y
5	A,C,D,E,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y

Table 2 demonstrates variations in a second order general pentapeptide sub-library.

Table 2.

c	Varied amino acids
1	A,C,D,E,F,G,M,N,P,Q,R,S,T,V,W,Y
2	A
3	L
4	A,L,M,N,P,Q,R,S,T,V,W,Y
5	A,C,D,E,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y

Examples for the synthesis of both the first and the second order sub-libraries of general libraries were described in the original presentation of the "portioning-mixing" method (cited above) although the terms introduced here were obviously not used.

The number of amino acid units in peptides is denoted by n. Coupling positions (c) are numbered from the C-terminus to express the order of couplings in the synthesis. The symbol p is used to indicate the number of amino acids varied in a coupling position. In the case of normal libraries p is constant while in general libraries may vary depending on coupling position. Symbol k is used to indicate the order of a sub-library.

Amino acids are denoted by one letter symbols: A (alanine),

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C (cysteine), D (aspartic acid), E (glutamic acid), F (phenylalanine), G (glycine), H (histidine), I (isoleucine), K (lysine), L (leucine), M (methionine), N (asparagine), P (proline), Q (glutamine), R (arginine), S (serine), T (threonine), V (valine), W (tryptophane), Y (tyrosine).

The symbol of sub-libraries comprises one or more pairs of a figure and a capital letter. The figure indicates the non-varied coupling position, while the letter is the symbol of the amino acid occupying it. The symbols of the first order sub-library of Table 1 and of the second order sub-library of Table 2 are 2E and 2A3L, respectively.

The number of peptides (N) in a standard peptide library depends on the number of residues: $N=20^n$, and that in one of its sub-library – in addition to n – depends on the order, too: $N=20^{(n-k)}$. Sub-libraries are classified according to the relative coupling positions of the non varied amino acids. For example, 2E and 2A3L are c 2 type first order and c 2,3 type second order sub-libraries, respectively. The number of types of sub-libraries (T) depends on both the order and the number of residues, and can be expressed by a simple formula.

$$T = \binom{n}{k}$$

For example, there are 10 types of second order pentapeptide sub-libraries which can be classified into two groups (Table 3):

Table 3.

vicinal types:	c	1,2	2,3	3,4	4,5
disjunkt types:	c	1,3	2,4	3,5	
	c	1,4	2,5		
	c	1,5			

Collections of sub-libraries of the same order are called *sub-kits*. The *first order sub-library sub-kits*, alternatively named *first sub-kits* comprise all possible first order sub-libraries of a parent peptide library. The first order sub-library sub-kit (Table 4) belonging to a standard pentapeptide library has 100 components.

Table 4.

1A,1C,1D,1E,1F,1G,1H,1I,1K,1L,1M,1N,1P,1Q,1R,1S,1T,1V,1W,1Y

2A,2C,2D,2E,2F,2G,2H,2I,2K,2L,2M,2N,2P,2Q,2R,2S,2T,2V,2W,2Y

3A,3C,3D,3E,3F,3G,3H,3I,3K,3L,3M,3N,3P,3Q,3R,3S,3T,3V,3W,3Y

4A,4C,4D,4E,4F,4G,4H,4I,4K,4L,4M,4N,4P,4Q,4R,4S,4T,4V,4W,4Y

5A,5C,5D,5E,5F,5G,5H,5I,5K,5L,5M,5N,5P,5Q,5R,5S,5T,5V,5W,5Y

Another sub-kit can be derived by combining those first order sub-libraries which carry identical amino acids in their non-varied coupling position (and are found in a column of Table 4). This sub-kit is named *amino acid tester sub-kit*.

$$\Sigma A,\Sigma C,\Sigma D,\Sigma E,\Sigma F,\Sigma G,\Sigma H,\Sigma I,\Sigma K,\Sigma L,\Sigma M,\Sigma N,\Sigma P,\Sigma Q,\Sigma R,\Sigma S,\Sigma T,\Sigma V,\Sigma W,\Sigma Y$$

A *second order sub-library sub-kit* comprises all possible second order sub-libraries of a given type or a collection of these. A 2,3 type second order sub-library sub-kit belonging to a standard pentapeptide library has 400 components (partially listed in Table 5. The vicinal type (see Table 3) second order sub-library sub-kit (belonging to the same parent library), which is particularly important in practice, has 1600 components. A collection of higher order sub-library sub-kits of a parent

Table 5.

2A3A,2C3A,2D3A,2E3A,2F3A,2G3A,.....,2R3A,2S3A,2T3A,2V3A,2W3A,2Y3A

2A3C,2C3C,2D3C,2E3C,2F3C,2G3C,.....,2R3C,2S3C,2T3C,2V3C,2W3C,2Y3C

2A3D,2C3D,2D3D,2E3D,2F3D,2G3D,.....,2R3D,2S3D,2T3D,2V3D,2W3D,2Y3D

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2A3V,2C3V,2D3V,2E3V,2F3V,2G3V,.....,2R3V,2S3V,2T3V,2V3V,2W3V,2Y3V

2A3W,2C3W,2D3W,2E3W,2F3W,2G3W,.....,2R3W,2S3W,2T3W,2V3W,2W3W,2Y3W

2A3Y,2C3Y,2D3Y,2E3Y,2F3Y,2G3Y,.....,2R3Y,2S3Y,2T3Y,2V3Y,2W3Y,2Y3Y

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peptide library is named *second sub-kit*. Collections of different order sublibrary sub-kits used in sequence determinations are called *sub-library kits*.

Description of the invention

The object of the present invention is a kit of peptide mixtures for determination of the amino acid sequences of bioactive peptides in a parent peptide library comprising a "first sub-kit" which consists of a complete set of first order sub-libraries of said parent peptide library and a "second sub-kit" of peptide mixtures consisting of a complete set of vicinal and disjunct type second order and possibly higher order sub-libraries of the same parent peptide library or a partial library (occurrence library) of the same parent peptide library and possibly also comprising an amino acid tester sub-kit consisting of combined components of the "first sub-kit". In screening experiments done with a series of properly selected sub-libraries, sufficient information can be gathered to determine the sequences of the bioactive peptides.

A preferred embodiment of our invention is the kit wherein the components of the first sub-kit are special partial libraries of the parent peptide library in which the same amino acid occupies a certain position in all peptides while in the rest of positions the amino acids are varied like in the parent peptide library. If a screening experiment is carried out with a first order sub-library, and the response is negative, it means, that the amino acid occupying the non-varied coupling position does not occur in that position of the bioactive peptides. If the response is positive, on the other hand, the amino acid in question does occur in the non-varied position in at least some of the active peptides.

Components of the second sub-kit are special partial libraries of the parent peptide library in which two positions are occupied by the same two amino acids in all peptides while in the rest of positions the amino acids are varied like in the parent peptide library. Since the second order sub-libraries have two non varied positions they can be used to check the occurrence of a pair of amino acids in two coupling positions. Both the vicinal and disjunct type representatives are important which can be used

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like dominoes in assembling the amino acid sequences of the bioactive peptides. Higher order sub-library sub-kits may also be included into the second sub-kit.

According to the invention components of the amino acid tester sub-kit are combined mixtures of all components of the first sub-kit in which the same amino acid occupies the non-varied position. The result of a screening experiment executed with a component of the amino acid tester sub-kit shows whether or not a certain amino acid occurs in any position of the bioactive peptides.

The number of amino acid residues of the peptides of parent peptide libraries might be preferably in the range of 2 to 25. Other ranges may be used as well.

According to the invention all kinds of amino acids can be used as subunits of peptides in the synthesis of the kit, like D-amino acids, unusual amino acids and/or derivatives of amino acids. The invention also refers to kits comprising mixtures of free or support-bound peptides.

The number of components in sub-library kits - and as a consequence the number of screening tests to be done - is in some cases quite considerable. Taking this in consideration we introduce in this invention labeling of sub-libraries. If some or all sub-libraries of a kit or sub-kit are labeled, the number of its components (and consequently the number of experiments in screening) can thoroughly be reduced, since the labeled sub-libraries can be mixed. The labels make the mixed sub-libraries distinguishable. For example, if 5 markers are used assigned to label coupling positions in the sub-kit demonstrated in Table 4, then sub-libraries of each column can be mixed resulting in 20 components instead of 100. Application of even a single marker in support bound kits (taking into consideration the unlabeled components) halves the number of components. The use of 20 markers assigned to amino acids makes possible to mix the components found in each row of Table 4 leading to only 5 components. By applying 20 markers the 400 components indicated in Table 5 can be replaced by 20 ones. The use of label carrying kits is advised when the kits are screened by binding experiments. Labeling can be applied in both support bound and free peptide kits. The applied colors have to be distinguished by eye or a suitable instrument (e.g. image processor). Among others the preferred colors may be: black, blue, green, cyan, red,

brown, light blue, light green, light cyan, light red, yellow.

Thus, according to our invention, it is an advantageous form of kits of free peptides in which color and/or fluorescent marker groups are attached to the C- and/or N-termini of peptides.

In the case of support-bound kits, addition to color or fluorescence, the size of otherwise uniform size beads or the specific gravity of the beads can also be used as markers in the synthesis of labeled support bound kits. Glass and polymer beads, for example, can easily be distinguished as a consequence of the difference in their specific gravity.

We have found that kits are preferable whereby the components of the kits are mixtures of sub-libraries attached to solid supports differing in color and/or fluorescence and/or specific gravity and/or the size of the beads, where the color and/or the fluorescence and/or the specific gravity and/or the size of the beads refer to (i) the coupling position of the non-varied amino acid, (ii) the amino acid occupying the non-varied position (iii) both the non-varied amino acid and its position, thus making the mixed sub-libraries distinguishable.

A further embodiment of our invention are the processes for the synthesis of the above peptide sub-library kits. The processes were designed so as to use the minimum number of coupling steps in the synthesis and they comprise combination and/or repetition of at least one of the operations described below (Operations Op/i to Op/xv).

Operations Op/i to Op/xv.

(i) Portioning into p equal portions of a solid support or an amino acylated support or a mixed amino acylated support or a support carrying peptides or a support carrying a mixture of peptides where p is the number of amino acids varied in the parent peptide library in a given coupling position.

(ii) Removal of the terminal amino-protecting group then coupling a different protected amino acid to each of p portions.

(iii) Mixing p portions.

(iv) Removal of $1/(n-r+1)$ part of each of p portions giving samples B, and the residues which are named (if any remaining) samples A, where r and n are the number of amino acid residues already attached to the support and the number of amino acid residues

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in the peptides of the parent peptide library, respectively.

(v) Execution of operations (i) to (iii) on each of p samples.

(vi) Execution of operations (i)-(ii) on each of p portions.

(vii) Two sets of B-samples, each set comprising p samples, are combined in pairs containing the same amino acid residue in the non-varied position of the peptides to form p combined samples, named B-samples.

(viii) Coupling a different protected amino acid to each of p portions.

(ix) Execution of operations (i) to (iii) on one sample.

(x) Submission of each of p portions to removal of R^2 protecting group of the support and to coupling with a different color or fluorescent marker.

(xi) Removal of terminal amino-protecting group and coupling a different color or fluorescent marker to each of p portions.

(xii) Portioning of each of p equal samples into p equal portions.

(xiii) Removal of $1/(n-r)$ part of each of p portions otherwise the same as operation (iv).

(xiv) Submission of each of p portions to removal of R^2 protecting group of the support and to coupling with a different color or fluorescent marker.

(xv) Removal of $1/(n-r-1)$ part of each of p portions, otherwise the same as operation (iv).

According to a preferable embodiment of our invention there is provided a process   for the synthesis of first sub-kit which comprises unlabeled free peptides in which the process is started with p equal samples of N-protected O-aminoacyl support, all samples differing in the aminoacyl residue.

(i) First, the samples are submitted to operation Op/iv, then samples A are consecutively submitted to operations Op/iii, Op/i and Op/ii and the resultant mixtures are used as starting samples in the next cycle of operations.

(ii) Samples B are submitted $(n-r)$ times to operation Op/v then the samples are taken apart.

(iii) The cycle of operations (i) to (ii) of this procedure is

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repeated (n-2) time.

Finally all samples are submitted to deprotection and cleavage from the support.

A process   for preparing a first sub-kit of peptides bound to unmarked support in which a sample of solid support, carrying blocked primary amino groups attached through a spacer, is first submitted to deprotection, operations Op/i and Op/viii, then the samples are treated according operations (i) to (iii) of the previous process and finally all samples are submitted to deprotection.

We also introduce a process   for preparing a first sub-kit of free peptides carrying color or fluorescent labels at their C-terminus assigned to the amino acid residues occupying the non-varied positions.

(i) The process is started with p equal samples of polymer or glass supports comprising N-protected O-aminoacyl groups wherein the aminoacyl group is a color or fluorescent marker differing in color or fluorescence of the label of the support.

(ii) The samples are submitted to operation Op/xiii.

(iii) Samples A (if any remaining) are submitted to operation Op/v then are used as starting samples in the next cycle of operations.

(iv) Samples B are submitted to operation Op/ii, then mixed and submitted consecutively (n-r) times to operation Op/ix then the sample is taken apart.

(v). Operations (ii) to (iv) of this procedure are repeated (n-1) times.

(vi) All samples are submitted to deprotection and cleavage from the support.

According to our invention we describe a process for synthesizing a first sub-kit of a normal parent peptide library bound to color and/or fluorescent support where the color, or the fluorescence, or the size or the specific gravity of the beads or combinations of these characteristics are assigned to the amino acid residues occupying the non-varied position.

(i) The process is started with p equal samples of polymer and glass supports comprising free or protected primary amino groups wherein the beads are colored or fluorescent, differing in at least

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one of the mentioned characteristics.

(ii) The samples are submitted to operations (ii) to (v) of the previous process and finally all samples are submitted to deprotection.

In order to further enhance the economy of the synthesis of labeled sub library sub-kits, special solid supports were devised and introduced in this invention and methods are described for their use in the synthesis of labeled support-bound sub-kits and sub-kits of free peptides carrying the label at their C-terminus. Our experiences clearly show that much labour can be saved in the synthesis of sub-kits if the synthesis is started with unlabeled support and the labels are introduced right before or right after building into peptides the non-varied amino acids. The solid supports (one for support bound kits and another one for kits of free peptides) devised for this purpose contain two kinds of protected primary amino groups. The two kinds of protecting groups can be removed independently. One of the protecting groups is removed to free the site of peptide synthesis the other one to allow coupling with the marker molecule. The color or fluorescent molecules applied as markers need to carry a functional group - among other possibilities a free carboxyl group - which make possible attachment to a free amino group. The advantages of this "in the course labeling" becomes clear if one compares to each other some characteristics of the three kinds of syntheses: (1) synthesis of unlabeled first sub-kits, (2) synthesis on labeled supports and (3) synthesis on unlabeled support with "in the course introduction" of the label. When (1) is carried out, after the introduction of the non-varied amino acids the samples have to be treated individually. From this stage of the synthesis the number of coupling cycles to be executed are multiplied by a factor identical with the number of the varied amino acids (say 19). In (2) the synthesis is started with say 19 different solid supports which have to be treated individually (and executing 19 times the same operations) until the non-varied amino acids are introduced. After this stage the samples can be mixed and the number of operations in every position is reduced by a factor of 19. If (3) is used, one can start the synthesis with one sample. After introduction of the non-varied amino acids, the samples can be mixed again to follow with one sample, since the labels are already introduced (right before or right after executing

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coupling with the non-varied amino acids). So reduced number of operations are needed both before and after the introduction of the non-varied amino acids.

As a preferable embodiment in our invention of the type of synthesis described in the previous paragraph we provide an economical process for the synthesis of a first sub-kit of free peptides carrying color or fluorescent labels at their C-terminus assigned to the amino acid residues occupying the non-varied positions. The process is started with one sample of polymer or glass supports comprising O-acylated hydroxymethyl groups corresponding to the following general formula where R^1 and R^2 are protecting groups, j, k, and r are integers 1 to 6.

$$R^1-NH-(CH_2)_j-CO-NH-(CH_2)_k-CH-CO-NH-(CH_2)_r-COO-CH_2-$$
$$R^2-HN$$
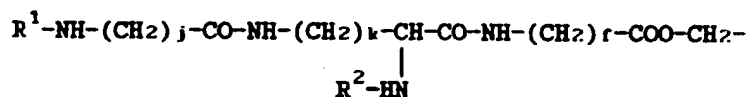
(i) The sample is submitted to operations Op/i and Op/ii.

(ii) The samples are then submitted to operation Op/iv.

(iii) Samples B are submitted to operation Op/x, then the samples are mixed and submitted ($n-r$) times to operation Op/ix and the sample is taken apart.

(iv) Samples A (if any remaining) are mixed then submitted to operations Op/i and Op/ii, then are used as starting samples in the next cycle of operations.

(v) The cycle of operations (ii) to (iv) of this procedure is repeated ($n-1$) times.

(vi) All samples are submitted to deprotection and cleavage from the support.

As a further preferable realization of our invention a very economical process is introduced for synthesizing a first sub-kit bound to color and/or fluorescent supports, using solid support comprising acylaminoalkyl groups corresponding to the following general formula 1 where R^1 and R^2 are protecting groups, j, k, and r are integers 1 to 6.

$$R^1-NH-(CH_2)_j-CO-NH-(CH_2)_k-CH-CO-NH-(CH_2)_r-$$
$$R^2-HN$$
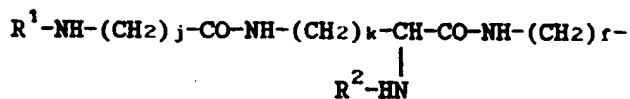
The color or fluorescent markers assigned to the amino acid residues

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occupying the non-varied position are attached to the support in the course of the peptide synthesis. The synthesis is started with one sample of support which is submitted to operations (i) to (v) of the previous procedure, finally, all samples are submitted to deprotection.

A process for synthesizing a first sub-kit of free peptides carrying color or fluorescent labels at their N-terminus assigned to the amino acid residues occupying the non-varied positions is a further preferable embodiment of our invention. N-terminal labeling is achieved by attaching color or fluorescent marker molecules (directly of *via* a spacer) to the N-terminal amino group of the peptides usually by acylation. The process is started with p equal samples of N-protected O-aminoacyl support, all samples differing in the aminoacyl residue.

(i) First, the samples are submitted to operation Op/iv.

(ii) Samples B are $(n-r)$ times consecutively submitted to operation Op/v. Then operation Op/xi is executed and after mixing, the sample is taken apart.

(iii) samples A (if any remaining) are consecutively submitted to operations Op/iii, Op/i and Op/ii and the resultant mixtures are used as starting samples in the next cycle of operations.

(iv) the cycle of operations (i) to (iii) of this procedure is repeated $(n-1)$ times then all samples are submitted to deprotection and cleavage from the support.

According to a preferable realization of our invention a process is introduced for synthesizing an amino acid tester sub-kit of free peptides comprising the following steps:

(i) The process is started with p equal samples of N-protected O-aminoacyl support, all samples differing in the acylamino residue.

(ii) First, the samples are submitted to operation Op/iv. On samples A (if any remaining) operations Op/iii, Op/i and Op/ii are consecutively executed then the samples are used in the next cycle operations. Samples B together with the resultant B samples of of the previous cycle (if executed) are submitted to operation Op/vii.

(iii) If $r < n$ samples B are submitted to operation Op/v otherwise are taken apart.

(iv) Cycle comprising operations (ii) to (iii) of this procedure are repeated $(n-1)$ times.

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(v) Finally, the resultant B samples are submitted to deprotection and cleavage from the support.

A process for synthesizing an amino acid tester sub-kit of peptides bound to unlabeled support has also been realized in our invention. The process is started with a sample of solid support, carrying blocked primary amino groups attached through a spacer. The sample is first consecutively submitted to deprotection and operations Op/i and Op/viii. Then the samples are treated according to operations (ii) to (iv) of the previous procedure. Finally the resultant B samples are submitted to deprotection.

Another preferable process in our invention is the synthesis of a sub-kit comprising all vicinal type second order sub-libraries of a parent peptide library of unlabeled soluble peptides, in which the process is started with p equal samples of O-acylamino support, all samples differing in the acylamino residue.

(i) The samples are submitted to operation Op/xi. Samples A (if any remaining) are consecutively submitted to operations Op/iii, Op/i, Op/ii then the resultant A samples are used in the next cycle of operations. Samples B are submitted to operation Op/vi then the unmixed resultant samples are one by one submitted to $n-r$ consecutive cycles of operations Op/i to Op/iii. All resultant samples originating from samples B are taken apart.

(ii) Cycle of operations (i) of this claim is repeated $n-2$ times.

(iii) All samples are submitted to cleavage and deprotection.

Our invention includes a process for synthesizing a sub-kit bound to unlabeled support comprising all vicinal type second order sub-libraries of a parent peptide library, in which a sample of solid support, carrying blocked primary amino groups attached through a spacer, is consecutively submitted to deprotection, operations Op/i and Op/viii, then the samples are treated according operations (i) to (ii) of the previous procedure, and finally, all samples are submitted to deprotection.

According to a preferable embodiment of our invention, a process is provided for synthesizing a sub-kit comprising all vicinal type second order sub-libraries of a parent peptide library of soluble peptides, carrying color or fluorescent labels at their C-terminus, assigned to the

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amino acid residue occupying the lower coupling position of the two vicinal non-varied positions, in which the process is started with p equal samples of solid support comprising N-protected O-aminoacyl groups wherein the aminoacyl group is a color or fluorescent marker, differing in color or fluorescence of the label of the support.

(i) first, operation Op/xv is executed. Samples A (if any remaining) are submitted to operation Op/v then the resultant A samples are used in the next cycle of operations. Samples B are consecutively submitted to operations Op/ii, Op/iii), Op/i) and Op/ii), then the resultant samples are submitted to $n-r$ consecutive cycles of operation Op/v) then the samples are taken apart.

(ii) cycle of operations (i) of this procedure is repeated ($n-2$) times.

(iii) Finally all samples are submitted to cleavage and deprotection.

A process for synthesizing a sub-kit comprising all vicinal type second order sub-libraries of a normal parent library of peptides bound to color or fluorescent supports, when the color or fluorescence of the support is assigned to the amino acid residue occupying the lower coupling position of the two vicinal non-varied positions is a further preferable embodiment of our invention. The process is started with p equal samples of polymer and glass supports comprising free or protected primary amino groups, wherein the beads are colored or fluorescent, differing in color or fluorescence of the label of the support. The samples are submitted to operations (i) to (ii) of the previous procedure, finally all samples are submitted to deprotection.

As an economical embodiment of our invention we introduce a process for synthesizing a sub-kit comprising all vicinal type second order sub-libraries of a normal parent library of peptides bound to color or fluorescent supports, when the color or fluorescence of the support is assigned to the amino acid residue occupying the lower coupling position of the two vicinal non-varied positions, and in which the process is started with a single deprotected sample of polymer and glass support comprising acylaminoalkyl groups corresponding to the following general formula 1 where R^1 and R^2 are protecting groups, j, k, and r are integers 1 to 6.

- 16 -
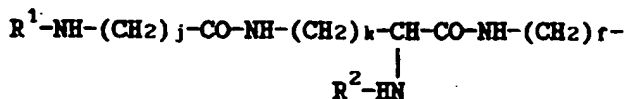
$$R^1\text{-NH-(CH}_2)_j\text{-CO-NH-(CH}_2)_k\text{-CH-CO-NH-(CH}_2)_r\text{-}$$
$$R^2\text{-HN}$$

The color or fluorescent labels are attached to the support in the course of the peptide synthesis.
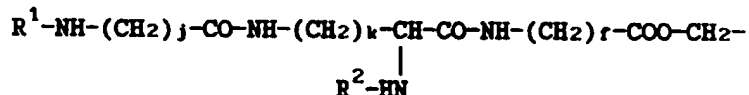
(i) The sample is submitted to operations Op/i and Op/ii.

(ii) The samples are submitted to operation Op/xiii. Samples A (if any remaining) are consecutively submitted to operations Op/iii, Op/i, Op/ii then the resultant A samples are used in the next cycle of operations. Samples B are submitted to operation Op/x, then mixed and submitted to operations Op/i and Op/ii then the resultant samples are submitted to $n-r$ consecutive cycles of operation Op/v then the samples are taken apart.

(iii) Cycle of operations (ii) of this procedure is repeated $n-2$ times.

(iv) Finally all samples are submitted to deprotection.

According to our invention we describe an economical process for the synthesis of a sub-kit comprising all vicinal type second order sub-libraries of a normal parent library of peptides bound to color or fluorescent supports, when the color or fluorescence of the support is assigned to the amino acid residue occupying the lower coupling position of the two vicinal non-varied positions. The process is started with a single deprotected sample of solid support comprising O-acylated hydroxymethyl groups corresponding to the following general formula where R^1 and R^2 are protecting groups, j, k, and r are integers 1 to 6

$$R^1\text{-NH-(CH}_2)_j\text{-CO-NH-(CH}_2)_k\text{-CH-CO-NH-(CH}_2)_r\text{-COO-CH}_2\text{-}$$
$$R^2\text{-HN}$$

and the color or fluorescent labels are introduced in the course of the peptide synthesis. The sample is submitted to operations (i) to (iii) of the previous procedure then all samples are submitted to deprotection and cleavage.

According to our invention, determination of the amino acid sequences of the bioactive peptides of a parent peptide library can be accomplished in three stages by using the domino strategy. There are no special

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requirements concerning the screening experiments used.

(i) First a screening experiment is carried out with all components of the amino acid tester kit. The positive answers show which amino acids occur in the active peptides regardless of their coupling position. The list of these amino acids is named *amino acid occurrence list*.

(ii) In the second stage, the screening experiments are done with a properly selected set of components of the first sub-kit to determine the coupling positions of the amino acids of the amino acid occurrence list. If, for example, alanine is present in the amino acid occurrence list of a pentapeptide parent library, one has to do screening experiments – among others – with the following first order sub-libraries: 1A, 2A, 3A, 4A and 5A. The result of these experiments show which amino acids vary in the different coupling positions of the bioactive peptides. These amino acids together with their coupling positions define a partial library of the parent one, named *occurrence library*. All bioactive peptides are present in the occurrence library, together with inactive ones but the total number of components is highly reduced relative to that of the parent library.

(iii) The third stage comprises determination of the sequences by use of the selected components of the second sub-kit. To exemplify this, let's suppose that the amino acid variations in the coupling positions 1 and 2 of the occurrence library are A, G and P, T, respectively. One has to determine whether P or T or both follow A, and, whether P or T or both follow G in different active sequences. Screening tests with 1A2P, 1A2T, 1G2P and 1G2T answer the questions. Then the amino acids varied in coupling positions 2,3 and 3,4 etc. of the occurrence library have to be similarly examined. Two different approaches can be followed in these experiments: (a) using selected components of the presynthesized second sub-kit of the parent peptide library or (b) synthesizing and using the second sub-kit the occurrence library.

If labeled support-bound sub-library kits are screened one has to observe the color or/and size etc. of the beads which bind macromolecules or cells. When labelled soluble kits are screened the gained color or/and

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fluorescence of the examined macromolecules, cells or tissues guides the experimenter in determining which component of the kit binds to the target.

Polymer and glass supports comprising free or protected primary amino groups, wherein the beads are colored or fluorescent are important objects of our invention. Color or fluorescent supports can be prepared by coupling a color or fluorescent marker molecule (and a spacer) to the surface of the beads. Those molecules can be used for this purpose which carry a functional group for attachment to the support - preferably a carboxyl group - and have another functional group - preferably an amino group - for coupling to the peptides or to a spacer. These color or fluorescent amino acids form a bridge between the solid support and the peptides.

Polymer and glass supports, comprising acylaminoalkyl groups corresponding to the following general formula where R^1 and R^2 are protecting groups, j, k, and r are integers 1 to 6, are important objects

$$R^1-NH-(CH_2)_j-CO-NH-(CH_2)_k-CH-CO-NH-(CH_2)_r-$$
$$R^2-HN$$
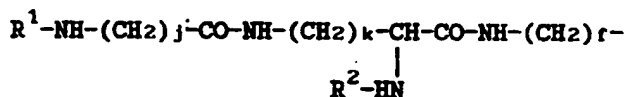
of our invention since they make possible introduction of the color or fluorescent label in the course of the synthesis of bound and labeled sub-library sub-kits. The built in marker molecules will form a bridge between the beads and the peptides to be synthesized.

Solid supports, carrying N-protected O-aminoacyl groups wherein the aminoacyl group is a color or fluorescent marker, are use in synthesis of sub-library kits comprising peptides labeled at their C-terminal part with color or fluorescent markers. The markers are color or fluorescent amino acid type molecules which form a bridge between the surface of the beads and the synthesized peptides and they remain attached to the peptides when the peptides are removed from the support at the end of the synthesis.

We also introduce solid supports comprising O-acylated hydroxymethyl groups corresponding to the following general formula where R^1 and R^2 are protecting groups, j, k, and r are integers 1 to 6.

$$R^1-NH-(CH_2)_j-CO-NH-(CH_2)_k-CH-CO-NH-(CH_2)_r-COO-CH_2-$$
$$R^2-HN$$
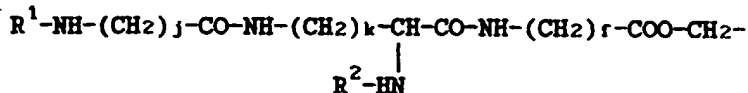
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We also introduce in our invention a special azo dye 4-[4-[N-Ethyl-N-[3-[tert-butyloxy-carbonyl]aminopropyl]amino]phenylazo]-benzoic acid (EPAP) and O-acylated as well as N-acylated polymer or glass supports derived by its use.

Advantages of the invention. The sub-library kits introduced in this invention may be regarded as new, generally applicable tools in screening of peptide mixtures. The "domino strategy" based on these kits is very economical and involves no condition of any kind concerning the method used in the screening experiments. Furthermore, support bound or free sub-library kits are promising equally good results, and the constituent amino acids are by no means restricted to the common natural ones. On the contrary, our synthetic and screening strategy can be applied to mixtures of any other kinds of bio-oligomers that can be synthesized by the solid phase method.

Support bound mixtures can be screened without the use of expensive sequenators and the method is independent of the current state of art of sequence determination of peptides incorporating unusual amino acids. Moreover, since no degradation is needed in sequence determinations the components of the kits are easily regenerated opening the possibility for a very economical multiple usage.

In screening of free peptide mixtures the use of sub-library kits and the "domino strategy" makes possible to determine the sequence of bioactive peptides in less screening steps than other methods do. Moreover, since no peptide synthesis is needed in determinations the method can easily be used by biologists lacking the special skills of peptide chemists.

The described syntheses of unlabeled sub-library kits are optimized so to use the less possible number of coupling cycles. Further reduction in the number of coupling cycles is achieved by using labeled polymers in the synthesis. The economy of the synthesis is even more increased by using special supports designed to allow introduction of the labels in the course of peptide synthesis.

The following examples are intended to demonstrate - but by no means limit - our invention.

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Examples

Example 1
N-Ethyl-N-[3-[tert-butyloxycarbonyl]aminopropyl]aniline
N-Ethyl-N-[3-aminopropyl]aniline (S.L.Shapiro et al., J.Amer.Chem. Soc. 1959, 81, 3083-3088) (1.78 g; 10 mmol) is reacted with di-tert-butyl dicarbonate according to L.Moroder et al. (Hoppe Seyler's Z.Physiol.Chem. 1976, 357, 1651-1653) to yield an oily product (90%).

Example 2
4-[4-[N-Ethyl-N-[3-[tert-butyloxycarbonyl]aminopropyl]amino]-
phenylazo]benzoic acid (EPAP)
4-Aminobenzoic acid (1.23 g; 9 mmol) dissolved in a mixture of ethanol (25 mL) and cc. hydrochloric acid (5 mL) is reacted with sodium nitrite (0.62 g; 9 mmol), the temperature being kept between 0° and 5°C. To the ice-cold solution of the diazonium salt, sodium acetate (4.1 g) then N-ethyl-N-[3-[tert-butyloxycarbonyl]-aminopropyl]aniline (Example 1) (2.5 g; 9 mmol) in ethanol (6 mL) are added. The mixture is stirred for 4 h at room temperature then allowed to stand overnight. The brick-red precipitate is filtered, washed with water and dried. The crude product is recrystallized from toluene to yield 2.4 g (62%).

Example 3
Assortment of polymer resin beads by size
Aminomethylated styrene-divinyl benzene (99:1) copolymer beads (10 g) (Mitchell et al., J.Amer.Chem.Soc. 1976, 98, 7357-7362; 1 meq N/g; 200-400 mesh [0.038-0.075 mm]) are sieved by using an upper and a lower sieve, 230 mesh (0.063 mm) and 325 mesh (0.045 mm), resp. The sieving results in three fractions of beads: 1. fine (325-400 mesh, 0.038-0.045 mm; 2 g), 2. medium (230-325 mesh, 0.045-0.063 mm; 5 g), 3. coarse (200-230 mesh, 0.063-0.075 mm; 3 g).

Example 4
Coarse Boc-Gaba-aminomethyl resin beads
Coarse aminomethyl resin beads (Example 3; 1 g) are swollen in dichloromethane (DCM), filtered, and a mixture of ethyl diisopropyl amine (EDIA) and DCM (1:9, v/v) (10 mL) is added. The mixture is shaken for 5 min, filtered, and the treatment with the former reagent solution is repeated. The mixture is allowed to stand for 15 min then filtered. To a

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solution of 0.81 g (4 mmol) Boc-γ-aminobutyric acid (Boc-Gaba) in DCM (5 mL), 1-hydroxybenzo- triazole (HOBt) (0.8 g; 5.2 mmol) then diisopropyl carbodiimide (DIC) (0.62 mL; 4 mmol) is added. The aminomethyl resin is suspended in this solution. The mixture is shaken for 3 h at room temperature, then the resin is filtered and washed (4x DCM, 2x ethanol [EtOH]). The beads which give negative test with ninhydrin (Kaiser et al., Anal.Biochem. 1970, 34, 595-598) are dried over P2O5.

Example 5

Fine Boc-Gaba-EPAP-aminomethyl resin beads

Fine aminomethyl resin beads (Example 3; 1 g) are swollen in DCM, filtered, and a mixture of EDIA and DCM (1:9, v/v) (10 mL) is added. The mixture is shaken for 5 min, filtered, and the washing with the former reagent solution is repeated. The mixture is allowed to stand for 15 min then filtered. To a solution of 1.70 g (4 mmol) EPAP (Example 2) in dimethyl formamide (DMF) (30 mL), HOBt (0.8 g; 5.2 mmol) then DIC (0.62 mL; 4 mmol) is added. The aminomethyl resin is suspended in this solution. The mixture is shaken for 3 h at room temperature. The red resin beads are filtered and washed (3x DMF, 3x DCM), filtered and a mixture of DCM and trifluoroacetic acid (TFA) (2:1, v/v) (10 mL) is added. The mixture is shaken for 5 min, filtered, again DCM/TFA mixture (10 mL) added and, after shaking for 30 min, the mixture is filtered, washed with DCM, MeOH, DCM, MeOH, 2x DCM. The resin is treated with EDIA and, after washing, with Boc-Gaba, HOBt and DIC according to Example 4.

Example 6

Nε-[Boc-Gaba]-Nα-Fmoc-L-Lysyl aminomethyl resin beads
(BGFL-aminomethyl resin beads)

Coarse aminomethyl resin beads (Example 3; 1 g) are treated as described in Example 4. The difference is that instead of Boc-Gaba, Nα-Fmoc-Nε-Boc-L-lysine (1.87 g; 4 mmol) is used. After coupling, the resin is reacted with DCM/TFA mixture (Example 5) then with EDIA and, after washing, with Boc-Gaba, HOBt and DIC according to Example 4.

Example 7

EPAP-Hydroxymethyl resin

Chloromethylated styrene-divinyl benzene (99:1) copolymer beads (Bio-Rad; 200-400 mesh; 1.26 meq Cl/g) (1 g) are swollen in DMF (30 mL) then EPAP (Example 2) (1.53 g; 3.6 mmol) and potassium fluoride (KF) (0.3 g) are

added. The mixture is stirred for 24 h at 50° then washed with DMF, DCM and EtOH. The red beads are dried over P2O5.

Example 8

Nε-Boc-Nα-Fmoc-L-Lysyl-[4-aminobutyryl]-hydroxymethyl resin

(BFLG-hydroxymethyl resin)

Chloromethylated styrene-divinyl benzene (99:1) copolymer beads (see Example 7) (1 g) are swollen in DMF (30 mL) then Boc-Gaba (0.73 g; 3.6 mmol) and KF (0.3 g) are added. The mixture is stirred for 24 h at 50°, washed with DMF and DCM, filtered, and a mixture of DCM and TFA (2:1, v/v) (10 mL) is added. The mixture is shaken for 5 min, filtered, shaken with a new portion (10 mL) of DCM/TFA, filtered and washed with DCM, MeOH, DCM, MeOH and 2x DCM. The resin is treated with EDIA and, after washing, with Nα-Fmoc-Nε-Boc-L-lysine, HOBt and DIC as described in Example 4 (lysine derivative is used instead of Boc-Gaba).

Example 9

Mixing of resin beads

Portions of resin beads (including starting resins, aminoacyl- and peptidyl-resins) are swollen in a mixture of DCM and DMF (2:1, v/v), poured and rinsed into a common polyethylene (PE) vessel. Swelling and rinsing require 5 mL liquid/100 mg of resin sample. The resultant mixture is shaken for 15 min then is ready for immediate portioning.

Example 10

Portioning of resin beads

A resin sample swollen in DCM/DMF (Example 9) is portioned by volume, using a 5 mL PE measuring pipette, into the reaction vessels (PE tubes fitted with plastic frit).

Example 11

Coupling of a Boc-amino acid to the resin beads

A sample of one of polymer supports described in Examples 4-8 or an Nα-Boc-aminoacyl- or an Nα-Boc-peptidyl resin containing 0.1 mmol of protected α-amino groups is placed into the reaction vessel (see Example 10). During the forthcoming operations the resin is shaken with different reagent solutions or washing solvents and at the end of operations the liquids are removed by suction. The operations are:

A/ Swelling (in the case of dry resin sample): 2x3 mL DCM, 2x5 min.

B/ Removal of Boc-groups: DCM/TFA 2:1 (v/v) 3 mL, 2 min; the same mixture,

3 mL, 30 min. C/ Washing: DCM, MeOH, DCM, MeOH, DCM, DCM, 3 mL and 5 min each. D/ Deprotonation: DCM/EDIA 9:1 (v/v) 3 mL, 2 min; the same mixture, 3 mL, 8 min. E/ Washing: DCM 3x3 mL, 9 min each. F/ Preparation of active ester: Boc-amino acid (side chain protecting groups used: OBzl for Asp and Glu, Bzl for Ser, Thr and Tyr, Z for Lys, Tos for Arg and His) (0.4 mmol) is dissolved in DCM/DMF 3:1 (v/v) (2 mL) in a separate vessel (in the case of Boc-Gln, Boc-Asn, Boc(Tos)-Arg and Boc(Tos)-His, the solvent is 2 mL of DMF) and, with stirring, HOBt (0.08 g; 0.52 mmol) then DIC (0.062 mL; 0.4 mmol) are added. G/ Coupling: The solution of active ester (see F) is added to the resin (prepared in section E of this Example), the mixture is shaken for 3 h at room temperature. H/ Washing: DMF (3x) then DCM (3x), 3 mL and 5 min each.

Example 12

Removal of Fmoc-groups

Resins carrying 0.1 mmol of a peptide mixture prepared by the use of resins described in Examples 6 or 8 as starting materials, are swollen in DCM then DMF (2x3 mL, 3 min each), shaken with DMF/piperidine (1:1, v/v) (3 mL) for 20 min, filtered and washed with 2x3 mL of DMF, dioxane/water 1:1, DMF and DCM, 5 min each.

Example 13

Labeling of peptides bound to BGFL-aminomethyl resin beads

Resin sample carrying a peptide mixture prepared by the use of BGFL-aminomethyl resin (Example 6) as starting materials, with free α-amino groups on the linker lysine residue after removal of Fmoc-groups as described in Example 12, is subjected to operations described in Example 11 F-H. Instead of a Boc-amino acid, however, marker molecules like 5-dimethylamino-1-naphthalenesulfonyl-Gaba (Dns-Gaba), 7-hydroxy-coumarin 4-acetic acid (HCAA) and EPAP (see Example 2) are used.

Example 14

Labeling of peptides to be cleaved from BFLG-hydroxymethyl resin beads

Resin sample carrying a peptide mixture prepared by the use of BFLG-hydroxymethyl resin (Example 8) as starting material, with free α-amino groups on the linker lysine residue after removal of Fmoc-groups described in Example 12, is treated according to Example 13.

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Example 15

Labeling of peptides at their N-termini

Resin sample carrying a peptide mixture prepared by the use of Boc-aminoacyl resin esters as starting materials, is subjected to operations described in Example 11 B-E, then to operations of Example 11 F-H (coupling with one of marker molecules; see Example 13).

Example 16

Removal of protecting groups from resin-bound peptides

Resin-bound peptide mixtures prepared by the use of resin beads described in Examples 4, 5 and 6 as starting materials, are treated with a mixture of trifluoromethanesulfonic acid (TFMSA) and TFA according to Yajima et al. (J.Chem.Soc., Chem.Commun. 1974, 107-108), then washed with ether and MeOH, dried over P_2O_5.

Example 17

Cleavage of peptides from the resin

Resin (50 mg), carrying peptide mixtures prepared from resins described in Examples 7 or 8, or from commercially available Boc-aminoacyl resin esters, is treated with TFMSA/TFA (see Example 16), the resin is filtered off, then the acidic reaction mixture is added to dry ether (25 mL). The mixture is allowed to stand overnight at -20°C. The precipitate is collected by filtration, washed twice with ether and dried over KOH.

Example 18

Mixing, portioning, coupling

Samples of 19 different protected aminoacyl resins are mixed (Example 9) then portioned (Example 10) resulting in 19 new samples which are separately coupled with one of the following amino acid derivatives according to Example 11: Boc-Ala, Boc(Tos)-Arg, Boc-Asn, Boc-Asp(OBzl), Boc-Gln, Boc-Glu(OBzl), Boc-Gly, Boc(Tos)-His, Boc-Ile, Boc-Leu, Boc(Z)-Lys, Boc-Met, Boc-Phe, Boc-Pro, Boc(Bzl)-Ser, Boc(Bzl)-Thr, Boc-Trp, Boc(Bzl)-Tyr, Boc-Val.

Example 19

Synthesis of the first sub-kit of a free standard-C tetrapeptide library

/i/   2.0 mmol samples of the 19 kinds of Boc-amino acid resin esters are submitted to 3 consecutive cycles of operations described as A-cycle in the next paragraph of this Example.

/ii/   A-cycle: 0.5 mmol of each sample is removed and the 19 removed

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samples are taken apart for use in B-cycles. The residual parts of samples are subjected to operations described in Example 18 to produce 19 new samples.

/iii/ Each of the 3x19 samples removed in A-cycles is repeatedly submitted to the following B-cycles of operations until it attains the tetrapeptide size.

/iv/ B-cycle: Each sample is divided into 19 equal portions, one of the 19 common Boc-amino acids is coupled to each portion then the portions are mixed resulting in 19 samples.

/v/ The 19 final samples of /ii/ and the 3x19 final samples of /iii/ are cleaved from the support according to Example 17.

Example 20

Synthesis of the first sub-kit of a resin-bound standard-C tetrapeptide library

19 kinds of Boc-aminoacyl-Gaba-aminomethyl resin beads are prepared by the use of different Boc-amino acids and the resin described in Example 4, according to Example 11. 2.0 mmol samples of the resultant 19 resins are submitted to 3 consecutive A-cycles (Example 19/ii) then operations described in Example 19 iii and iv. The protecting groups from the 76 resin-bound final samples are removed according to Example 16.

Example 21

First sub-kit of a free standard-C tetrapeptide library carrying color- and/or fluorescence-labels at the C-termini of peptides starting from labeled resin esters

/i/ 19 kinds of color- and/or fluorescence-labeled resin esters (2.0 mmol each) are used as starting materials and submitted to 4 consecutive main cycles.

/ii/ Main cycle: 0.5 mmol of each resin sample is removed and taken apart as B-sample. Each of the remaining samples (A-samples; if any remaining) is submitted to the following operations: dividing into 19 equal parts, each part is coupled with a different Boc-amino acid according to Example 11 then the samples are mixed and taken apart for using as starting material in the next cycle. Each of B-samples is coupled with a different Boc-amino acid according to Example 11, then the samples are mixed. If the peptides are shorter then tetrapeptides, the sample is submitted to consecutive inner cycles until the tetrapeptide size is

attained.

/iii/ Inner cycle of the main cycle: The sample is divided into 19 parts, each part is coupled with a different Boc-amino acid then the samples are mixed.

/iv/ Finally the peptides of the 4 samples are cleaved from the support according to Example 17.

Example 22

First sub-kit of a resin-bound standard-C tetrapeptide library synthesized on beads differing from each other by their color, fluorescence and/or size

19 kinds of colorless (Example 4), colored (Example 5) and fluorescent derivatives of fine and coarse aminomethyl resin beads (Example 3) are used as staring materials and submitted to operations described in paragraphs /ii/ and /iii/ of Example 21. The protecting groups from the 4 final samples are removed according to Example 16.

Example 23

First sub-kit of a free standard-C tetrapeptide library carrying color or fluorescent labels at the C-termini of peptides (labeling in the course)

BFLG-hydroxymethyl resin (Example 8) is divided into 19 equal portions and each of them is submitted to operations described in Example 11 resulting in 19 different Boc-aminoacyl-BFLG-hydroxymethyl resins, 2.0 mmol of each.

/i/ The resultant resins are submitted to 3 consecutive E-cycles of the following operations.

/ii/ E-cycle: 0.5 mmol of each sample is removed. The 19 removed samples are taken apart for use in F-cycles. The residual parts of the samples are mixed together, divided into 19 equal parts, and each new part is coupled with a different Boc-amino acid.

/iii/ Fmoc-group is removed from each sample obtained after the last E-cycle according to Example 12 then each sample is coupled with a different color and/or fluorescence marker (see Example 14) and the samples are mixed together to produce one of the 4 final samples.

/iv/ Each of the sets of 19 samples removed and taken apart in the different E-cycles, is treated as described in /iii/ of this Example, then repeatedly submitted to the F-cycles of operations, described in the next paragraph, until it attains the tetrapeptide size and produce 3 final samples.

/v/   F-cycle: The sample is divided into 19 equal portions, each portion
is coupled with a different Boc-amino acid then the new portions are mixed
together.

/vi/   The peptides of the 4 final samples (originated in /iii/ and /iv/)
are cleaved from the support according to Example 17.

Example 24

Labeled first sub-kit of a resin-bound standard-C tetrapeptide library
(labeling in the course)

19 kinds of Boc-aminoacyl resin beads (2.0 mmol of each) are prepared
according to Example 11, using BGFL-aminomethyl resin beads (Example 6).
On these Boc-aminoacyl resins, operations described in Example 23 i to
23 v are executed. The protecting groups from the 4 final samples are
removed according to Example 16.

Example 25

First sub-kit of a free normal pentapeptide library carrying color labels
at the N-termini of peptides

/i/   2.0 mmol samples of 10 different   Boc-amino acid resin esters
(resin esters of Boc-Ala, Boc(Tos)-Arg, Boc-Asp-OBzl,   Boc-Glu(OBzl),
Boc-Gly, Boc(Z)-Lys, Boc-Phe, Boc-Pro, Boc(Bzl)-Ser and Boc(Bzl)-Tyr) are
submitted to 4 consecutive G-cycles of the following operations.

/ii/   G-cycle: 0.4 mmol of each sample is removed and taken apart for use
in H-cycles. The residual parts of samples are mixed together, divided
into 10 portions, then each portion is coupled with a   different
Boc-amino acid (Example 11). The resultant samples (except the remainder
produced in the last cycle) are used as starting samples in the next
cycle.

/iii/ Each of the sets of 10 samples removed in G-cycles is repeatedly
submitted to the following H-cycles of operations until it attains the
pentapeptide size.

/iv/   H-cycle: Each sample is submitted to the following operations:
dividing into 10 equal portions, one of the Boc-amino acids listed in /i/
of this Example is coupled to each portion, then the sets of 10 resultant
portions are mixed together.

/v/   The 10 remainder samples of the 4th G-cycle as well as the 4x10
samples produced in H-cycles are one by one coupled with Boc-Gaba
(likewise Example 11) resulting in 5 sets of 10 samples. Then each set is

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treated as follows: each of the 10 samples is coupled with one of different color markers as described in Example 15, then mixed together to produce 1 final sample.

/vi/ The final samples obtained in /v/ are cleaved from the support according to Example 17.

Example 26

Amino acid tester sub-kit of a free standard-C tripeptide library

/i/ 19 samples (numbered from 1 to 19) of 2.1 mmol Boc-amino acid resin esters - containing the amino acid residues in order of A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, Y - are submitted to 3 consecutive I-cycles of operations.

/ii/ I-Cycle: 0.7 mmol of each sample is removed (inheriting the number of the original sample) and the samples are taken apart for use as starting samples in operations described in paragraph /iii/. The residual parts of samples (if any remaining) are mixed together, divided into 19 numbered portions, then each portion is coupled with a different Boc-amino acid in order described in paragraph /i/. The resultant numbered samples are used as starting samples in the next I-cycle.

/iii/ Each of 19 samples removed in the first I-cycle is treated as follows: the sample is divided into 19 portions. Each portion is coupled with a different Boc-amino acid then mixed. In this way 19 new numbered samples are formed.

/iv/ Each of the 19 samples removed in the second I-cycle is united with its identically numbered pair obtained in paragraph /iii/ and the new sample is carrying the same number. Each of 19 united samples is treated as follows: the sample is divided into 19 new portions, and each portion is coupled with a different Boc-amino acid then mixed to one sample carrying the original number.

/v/ Each of the 19 samples obtained in /iv/ is united with the identically numbered sample removed in the third I-cycle to produce 19 final samples.

/vi/ The peptides of 19 final samples are cleaved from the support according to Example 17.

Example 27

Amino acid tester sub-kit of a resin-bound standard-C tripeptide library

19 samples (numbered from 1 to 19) of 2.1 mmol Boc-aminoacyl-Gaba-amino-

methyl resin (see Examples 4 and 11) - containing the amino acid residues
in order of A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, Y - are
submitted to 3 consecutive I-cycles of Example 26 then operations
described in Example 26 iii to 26 v are executed. The protecting groups
from the 19 resin-bound final samples are removed according to Example 16.

Example 28

Second order vicinal sub-library sub-kit of a free standard-C tetrapeptide
library

1.5 mmol samples of the 19 kinds of Boc-amino acid resin esters are
submitted to 3 consecutive main cycles of the following operations.

Main cycle: 0.5 mmol of each sample is removed; they are taken apart as
B-samples. The residual parts of samples (A-samples; if any remaining) are
mixed together, divided into 19 portions, each portion is coupled with a
different Boc-amino acid.

Each of the 19 B-samples is treated as follows: the sample is divided into
19 portions then each portion is coupled with a different Boc-amino acid
then the resultant 19x19 samples are submitted to the following inner
cycle of operations until they attain the tetrapeptide size.

Inner cycle: each of the 19x19 samples is treated as follows: divided into
19 parts. Each part is coupled with one of the 19 Boc-amino acids then
mixed together (end of both cycles).

The peptides of the 3x361 final samples are cleaved from the support
according to Example 17 to obtain the kit of 1,2-, 2,3- and 3,4-types of
second order vicinal sub-libraries.

Example 29

Second order vicinal sub-library sub-kit of a resin-bound normal
tetrapeptide library

1.5 mmol samples of 10 different Boc-aminoacyl-GABA-aminomethyl
resins (prepared from resin described in Example 4 according to
Example 11; kinds of Boc-amino acid resin esters are listed in Example 25)
are treated as described in Example 28 with the following modifications:
10 kinds of samples are used instead of 19; the number of final samples is
3x100 instead of 3x361; the final samples are treated as described in
Example 16.

Example 30

Labeled second order vicinal sub-library sub-kit of a free normal
tetrapeptide library, synthesized on labeled supports

2.1 mmol samples of 9 kinds of color or fluorescence labeled resins (see
Example 7) are submitted to 3 consecutive main cycles of operations.

Main cycle: 0.7 mmol of each resin sample is removed and taken apart as
B-sample. The residual parts of samples (if any remaining) are named
A-samples. Each of A-samples is treated as follows: the sample is divided
into 9 portions, each portion is coupled with one of the 9 Boc-amino acids
(Boc-amino acids listed in Example 25 are used with the following
modifications: Boc-Pro is omitted; D-Phe is used instead of Phe). The
lengthened samples are mixed together to form a new sample used as one of
the starting samples in the next main cycle.

Each of B-samples is coupled with a different Boc-amino acid (see above),
the 9 new samples are mixed together, divided into 9 portions, each
portion is coupled with a different Boc-amino acid. The resultant 9
samples are consecutively submitted to the inner cycles of operations
until they attain the tetrapeptide size.

Inner cycle: Each of the 9 samples (if the peptides are shorter than
tetrapeptides) is treated as follows: the sample is divided into 9
portions and each portion is coupled with one of Boc-amino acids (see
above) then mixed. The mixed samples (if shorter than tetrapeptides) are
used as starting samples in the next inner cycle.

This is the end of both cycles.

The peptides of 3x9 final samples obtained are cleaved from the support
according to Example 17.

Example 31

Second order vicinal sub-library sub-kit of a normal tetrapeptide library
bound to color or fluorescent supports

2.1 mmol samples of 9 kinds of color or fluorescence labeled resins (see
Example 5) are treated according to Example 30 with the exception of the
last step in which the protecting groups are removed from the final
samples as described in Example 16.

Example 32

Labeled resin-bound second order vicinal sub-library sub-kit of a normal tetrapeptide library (labeling in the course)

21 mmol of BGFL-aminomethyl resin beads (Example 6) is divided into 10 equal parts. Each part is coupled with one of 10 Boc-amino acids (Boc-amino acids listed in Example 25 are used with a single modification: Boc-L-α-aminobutyric acid is applied instead of Boc-Pro). The resultant samples are submitted to 3 consecutive main cycles of operations.

Main cycle: 0.7 mmol of each resin sample is removed and taken apart as one of the B-samples. The residual parts of samples (A-samples; if any remaining) are mixed together then divided into 10 parts and each of them is coupled with a different Boc-amino acid (see above). The resultant samples are used as starting samples in the next main cycle.

Each of B-samples is treated as follows: the Fmoc-group is removed according to Example 12 then the sample is coupled with a color or fluorescence marker (see Example 14). The resultant B-samples are mixed together and divided into 10 portions. Each of new portions is coupled with a different Boc-amino acid. The resultant 10 samples are consecutively submitted to the inner cycles of operations until they attain the tetrapeptide size.

Inner cycle: Each of the samples (if the peptides are shorter than tetrapeptides) is treated as follows: divided into 10 equal portions, the portions are coupled with different Boc-amino acids (see above) then mixed. The samples (if the peptides are shorter than tetrapeptides) are used as starting samples in the next inner cycle.

This is the end of both cycles.

The protecting groups of the 3x10 final samples are removed according to Example 16.

Example 33

Second order vicinal sub-library sub kit of a free normal tetrapeptide library carrying color or fluorescent labels at the C-termini of peptides (labeling in the course)

21 mmol of BFLG-hydroxymethyl resin (Example 8) is treated according to Example 32 with the exception of the last step in which the peptides of the final samples are cleaved from the support as described in Example 17.

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Example 34

Screening of 4A first order standard-C tetrapeptide sub-library (4A4), bound to unlabeled support, with rat red blood cells

The experiment is carried out as recently described by Sebestyén et al. (Bioorg. Med. Chem. Letters 1993, 3, 413-418). 0.5 mg of 4A4 and 0.5 mL of red blood cell suspension (prepared from heparinized rat blood; the cells are washed and centrifuged 3 times before use to remove serum proteins) is kept at 0°C for 15 min, shaking gently several times, then pipetted into 10 mL Seglen's suspension buffer (Ssb). The diluted suspension is gently centrifuged (200 rpm, 3 min) to facilitate sedimentation of the beads. The excess of cells is removed by carefully pipetting out the supernatant. The sediment is diluted with 3 mL Ssb, pipetted into a Petri-dish and surveyed in an inverted microscope.

Although most of the beads are bare, some are completely covered with cells so screening test of 4A4 is considered positive.

Example 35

Screening with a support-bound standard-C tetrapeptide amino acid tester sub-kit.

All of the 19 components of the support-bound standard-C tetrapeptide amino acid tester sub-kit are submitted to the screening test described in Example 34. All tests resulting in formation of at leas some completely covered beads are considered positive and the amino acid appearing in symbol of the component giving positive result is included into the amino acid occurrence list.

Example 36

The use of the components of an unlabeled resin-bound first sub-kit of a standard-C tetrapeptide library in screening.

To the amino acid occurrence list, A, E, H, K, L, N, Y, the following components of the first order sub-library sub-kit described in Example 20 are selected for screening described in Example 34: 1A, 2A, 3A, 4A, 1E, 2E, 3E, 4E, 1H, 2H, 3H, 4H, 1K, 2K, 3K, 4K, 1L, 2L, 3L, 4L, 1N, 2N, 3N, 4N, 1Y, 2Y, 3Y, 4Y. The amino acids appearing in the symbol of the components giving positive results are entered - from position to position - into the list of amino acids varied in the occurrence library.

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Example 37

The use of the components of an unlabeled resin-bound second order
standard-C tetrapeptide sub-library sub-kit in screening.

In order to determine the sequences of the active peptides present in the
occurrence library demonstrated in Table 6 the following components of the

Table 6.

_____

1   A

2   E, K

3   L, N

4   H, Y

_____

second order sub-library sub-kit described in Example 29 (or synthesized
as the second order vicinal sub-library sub-kit of the occurrence library)
are submitted to the screening test (Example 34): 2E3L, 2E3N, 2K3L, 2K3N,
3L4H, 3L4Y, 3N4H, 3N4Y. The active components may be used as dominoes in
determining the sequences of the active peptides present in the occurrence
library as shown below.

        1A
         2E3L
           3L4H

                                        c 1   4
The deduced sequence is: AELH

Written in usual order: HLEA

In some cases testing with disjunct second order sub-libraries is also
needed.

Example 38

First sub-kit of a free general tripeptide library

/i/   1.0 mmol samples of each of Boc-amino acid resins derived from Ala,
Asn, Gly, Met, Pro and Val are used as starting materials. One-third of
each of 6 samples is removed and taken apart (B1-samples).

/ii/   The residual parts of each sample (A1-samples) are mixed then
portioned into 4 equal parts. Each part is coupled with one of the

following Boc-amino acids: Boc-Glu(OBzl), Boc-Leu, Boc(Z)-Lys and Boc-Phe.

/iii/ Half of each of the resultant 4 samples (see paragraph /ii/) is removed and taken apart (B2-samples). The residual parts of each sample (A2-samples) are mixed then divided into 10 equal parts. Each part is coupled with one of the Boc-amino acids listed in Example 25 to produce 10 final samples.

/iv/ Each B1-sample (see paragraph /i/) is treated as follows: divided into 4 equal parts and each part is coupled with one of Boc-amino acids listed in paragraph /ii/ then mixed.

/v/ Each of the resultant 4 samples (see paragraph /iv/) is treated as follows: divided into 10 equal parts and each part is coupled with one of Boc-amino acids listed in Example 25 then mixed.

/vi/ Each B2-sample (see paragraph /iii/) is treated as described in paragraph /v/.

/vii/ The 3x10 final samples obtained in paragraphs /iii/, /v/ and /vi/ are treated according to Example 17.

Example 39

Labeled second order 1,3 and 2,4 type disjunct sub-library sub-kit of a resin-bound normal tetrapeptide library. The labels refer to the lower non-varied coupling position (labeling in the course)

20 mmol of BGFL-aminomethyl resin beads (Example 6) is divided into 10 equal parts. Each part is coupled with one of 10 Boc-amino acids (Boc-amino acids listed in Example 25 are used with a single modification: Boc-L-α-aminobutyric acid is applied instead of Boc-Pro). The resultant samples are submitted to 2 consecutive main cycles of operations.

Main cycle: 1.0 mmol of each resin sample is removed and taken apart as one of the B-samples. The residual parts of samples (A-samples; if any remaining) are mixed together then divided into 10 parts and each of them is coupled with a different Boc-amino acid (see above). The resultant samples are used as starting samples in the next main cycle.

Each of B-samples is treated as follows: the Fmoc-group is removed according to Example 12 then the sample is coupled with a color or fluorescence marker (see Example 14). The resultant 10 samples are consecutively submitted to two inner cycles of operations.

Inner cycle: The resultant B-samples are mixed together and divided into 10 portions. Each of new portions is coupled with a different

Boc-amino acid. This is the end of main cycle and the inner cycle.

Each of the samples coming out from the second inner cycle (if the peptides are shorter than tetrapeptides) is treated as follows: divided into 10 equal portions, the portions are coupled with different Boc-amino acids (see above) then mixed. The protecting groups of the 2x10 final samples are removed according to Example 16.

CLAIMS

WHAT IS CLAIMED IS:

1. A kit of peptide mixtures for determination of the amino acid sequences of bioactive peptides in a parent peptide library comprising
a "first sub-kit" which consists of a complete set of first order sub-libraries of said parent peptide library and
a "second sub-kit" of peptide mixtures consisting of a complete set of vicinal and disjunct type second order and possibly higher order sub-libraries of the same parent peptide library or a partial library (occurrence library) of the same parent peptide library
and possibly also comprising an amino acid tester sub-kit ("third sub-kit"), consisting of combined components of the first sub-kit.

2. A kit according to claim 1 wherein the components of the first sub-kit are special partial libraries of the parent peptide library in which the same amino acid occupies a certain position in all peptides while in the rest of positions the amino acids are varied like in the parent peptide library.

3. A kit according to claim 1 wherein the components of the second sub-kit are special partial libraries of the parent peptide library in which the same two positions are occupied by the same two amino acids in all peptides while in the rest of positions the amino acids are varied like in the parent peptide library.

4. A kit according to claim 1 wherein the components of the amino acid tester kit sub-kit are combined mixtures of all those components of the first sub-kit in which the non-varied position is occupied by the same amino acid.

5. A kit according to any of claims 1 to 4 in which the number of amino acid residues of the peptides of parent peptide libraries is in the range of 2 to 25.

6. A kit according to any of claims 1 to 5 in which the amino acid subunits of peptides are L-amino acids, D-amino acids, unusual amino acids and/or derivatives of amino acids.

7. A kit according to any of claims 1 to 6 comprising mixtures of free or support-bound peptides.

8. A kit of free peptides according to any of claims 1 to 7 in which color and/or fluorescent marker groups are attached to the C- and/or N-termini of peptides.

9. A kit according to any of claims 1 to 7 in which the components of the kits are mixtures of sub-libraries attached to solid supports differing in color and/or fluorescence and/or specific gravity and/or the size of the beads, where the color and/or the fluorescence and/or the specific gravity and/or the size of the beads refer to (i) the coupling position of the non-varied amino acid, (ii) the amino acid occupying the non-varied position (iii) both the non-varied amino acid and its position, thus making the mixed sub-libraries distinguishable.

10. Process for the synthesis of peptide sub-library kits according to any of claims 1 to 9 which comprises combination and/or repetition of at least one of the following operations;

(i) portioning into p equal portions of a solid support or an amino acylated support or a mixed amino acylated support or a support carrying peptides or a support carrying a mixture of peptides where p is the number of amino acids varied in the parent peptide library in a given coupling position;

(ii) each of p portions are submitted to removal of the terminal amino-protecting group and coupling with a different protected amino acid;

(iii) mixing p portions;

(iv) removal of $1/(n-r+1)$ part of each of p portions giving samples B, and the residues which are named (if any remaining) samples A, where r and n are the number of amino acid residues already attached to the support and the number of amino acid residues

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in the peptides of the parent peptide library, respectively;

(v) execution of operations (i) to (iii) on each of p samples;

(vi) execution of operations (i)-(ii) on each of p portions;

(vii) two sets of B-samples, each set comprising p samples, are combined in pairs containing the same amino acid residue in the non-varied position of the peptides to form p combined samples, named B-samples;

(viii) coupling a different protected amino acid to each of p portions;

(ix) execution of operations (i) to (iii) on one sample;

(x) submission of each of p portions to removal of R^2 protecting group of the support and to coupling with a different color or fluorescent marker;

(xi) removal of terminal amino-protecting group and coupling a different color or fluorescent marker to each of p portions;

(xii) portioning of each of p equal samples into p equal portions;

(xiii) removal of $1/(n-r)$ part of each of p portions otherwise the same as operation (iv);

(xiv) submission of each of p portions to removal of R^2 protecting group of the support and to coupling with a different color or fluorescent marker;

(xv) removal of $1/(n-r-1)$ part of each of p portions, otherwise the same as operation (iv).

11. Process for the synthesis of first sub-kit according to any of claim 10 which comprises unlabeled free peptides in which the process is started with p equal samples of N-protected O-aminoacyl support, all samples differing in the aminoacyl residue;

(i) first, the samples are submitted to operation (iv) of claim 10, then samples A are consecutively submitted to operations (iii), (i) and (ii) of claim 10 and the resultant mixtures are used as starting samples in the next cycle of operations;

(ii) samples B are submitted $(n-r)$ times to operation (v) of claim 10 then the samples are taken apart;

(iii) the cycle of operations (i) to (ii) of this claim is

repeated (n-2) times;

(iv) all samples are submitted to deprotection and cleavage from the support.

12. A process according to claim 10 for preparing a first sub-kit of peptides bound to unmarked support in which a sample of solid support, carrying blocked primary amino groups attached through a spacer, is first submitted to deprotection, operations (i) and (viii) of claim 10, then the samples are treated according operations (i) to (iii) of claim 11, and finally all samples are submitted to deprotection.

13. A process according to claim 10 for preparing a first sub-kit of free peptides carrying color or fluorescent labels at their C-terminus assigned to the amino acid residues occupying the non-varied positions;

(i) the process is started with p equal samples of solid supports comprising N-protected O-aminoacyl groups wherein the aminoacyl grop is a color or fluorescent marker differing in color or fluorescence of the label of the support;

(ii) the samples are submitted to operation (xiii) of claim 10;

(iii) samples A (if any remaining) are submitted to operation (v) of claim 10 then are used as starting samples in the next cycle of operations;
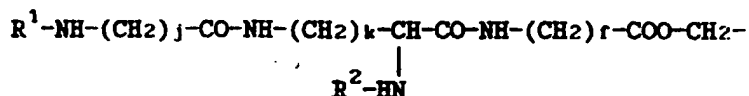
(iv) samples B are submitted to operation (ii) of claim 10, then mixed and submitted consecutively (n-r) times to operation (ix) of claim 10 then the sample is taken apart;

(v) operations (ii) to (iv) of this claim are repeated (n-1) times;

(vi) all samples are submitted to deprotection and cleavage from the support.

14. A process for synthesizing a first sub-kit of free peptides carrying color or fluorescent labels at their C-terminus assigned to the amino acid residues occupying the non-varied positions, in which the process is started with one sample of solid support comprising O-acylated hydroxymethyl groups corresponding to the following general formula where R^1 and R^2 are protecting groups, j, k, and r are integers 1 to 6;

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$$R^1-NH-(CH_2)_j-CO-NH-(CH_2)_k-CH-CO-NH-(CH_2)_r-COO-CH_2-$$
$$R^2-HN$$

(i) the sample is submitted to operations (i) and (ii) of claim 10;

(ii) the samples are then submitted to operation (iv) of claim 10;

(iii) samples B are submitted to operation (x) of claim 10, then the samples are mixed and submitted $(n-r)$ times to operation (ix) of claim 10 and the sample is taken apart;

(iv) samples A (if any remaining) are mixed then submitted to operations (i) and (ii) of claim 10, then are used as starting samples in the next cycle of operations;

(v) The cycle of operations (ii) to (iv) of this claim is repeated $(n-1)$ times;

(vi) all samples are submitted to deprotection and cleavage from the support.

15. A process for synthesizing a first sub-kit of free peptides carrying color or fluorescent labels at their N-terminus assigned to the amino acid residues occupying the non-varied positions, in which the process is started with p equal samples of N-protected O-aminoacyl support, all samples differing in the aminoacyl residue;

(i) first, the samples are submitted to operation (iv) of claim 10;

(ii) samples B are $(n-r)$ times consecutively submitted to operation (v) of claim 10, then to one operation (xi) of claim 10, and after mixing, the sample is taken apart;

(iii) samples A (if any remaining) are consecutively submitted to operations (iii), (i) and (ii) of claim 10 and the resultant mixtures are used as starting samples in the next cycle of operations;

(iv) the cycle of operations (i) to (iii) of this claim is repeated $(n-1)$ times;

(v) all samples are submitted to deprotection and cleavage from the support.

16. A process for synthesizing an amino acid tester sub-kit of free peptides comprising the following steps:

(i) the process is started with p equal samples of N-protected O-aminoacyl support, all samples differing in the acylamino residue;

(ii) first, the samples are submitted to operation (iv) of claim 10;

(iii) on samples A (if any remaining) operations (iii), (i) and (ii) of claim 10 are consecutively executed then the samples are used in the next cycle operations;

(iv) samples B together with the resultant B samples of the previous cycle (if executed) are submitted to operation (vii) of claim 10;

(v) if $r < n$ samples B are submitted to one operation (v) of claim 10 otherwise are taken apart;

(vi) cycle comprising operations (ii) to (v) of this claim are repeated (n-1) times;

(vii) finally, the resultant B samples are submitted to deprotection and cleavage from the support.

17. A process for synthesizing an amino acid tester sub-kit of peptides bound to unlabeled support comprising the following steps:

(i) the process is started with a sample of solid support, carrying blocked primary amino groups attached through a spacer;

(ii) The sample is first consecutively submitted to deprotection and operations (i) and (viii) of claim 10;

(iii) then the samples are treated according to operations (ii) to (vi) of claim 16;

(vi) finally the resultant B samples are submitted to deprotection.

18. A process for synthesizing a first sub-kit of a normal parent peptide library bound to color and/or fluorescent support where the color, or the fluorescence, or the size or the specific gravity of the beads or combinations of these characteristics are assigned to the amino acid residues occupying the non-varied position;

(i) the process is started with p equal samples of polymer and

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glass supports of different bead sizes comprising free or protected primary amino groups wherein the beads are colored or fluorescent, differing in at least one of the mentioned characteristics;

(ii) the samples are submitted to operations (ii) to (v) of claim 13;

(iii) finally all samples are submitted to deprotection.

19. A process for synthesizing a first sub-kit bound to color and/or fluorescent supports, using solid support comprising acylaminoalkyl groups corresponding to the following general formula where R^1 and R^2 are protecting groups, j, k, and r are integers 1 to 6

$$R^1-NH-(CH_2)_j-CO-NH-(CH_2)_k-CH-CO-NH-(CH_2)_r-$$
$$R^2-HN$$
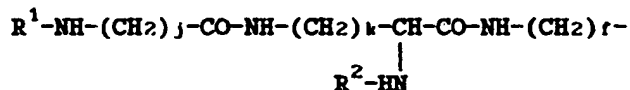
and the color or fluorescent markers assigned to the amino acid residues occupying the non-varied position are attached to the support in the course of the peptide synthesis;

(i) the synthesis is started with one sample of support;

(ii) the sample is submitted to operations (i) to (v) of claim 14;

(iii) finally, all samples are submitted to deprotection.

20. A process for synthesizing a sub-kit comprising all vicinal type second order sub-libraries of a parent peptide library of unlabeled soluble peptides, in which the process is started with p equal samples of O-acylamino support, all samples differing in the acylamino residue;

(i) the samples are submitted to operation (xiii) of claim 10;

(ii) samples A (if any remaining) are consecutively submitted to operations (iii), (i), (ii) of claim 10 then the resultant A samples are used in the next cycle of operations;

(iii) samples B are submitted to operation (vi) of claim 10 then the unmixed resultant samples are one by one submitted to $n-r$ consecutive cycles of operations (i) to (iii) of claim 10;

(iv) all resultant samples originating from samples B are taken apart;

(v) cycle of operations (i) to (iv) this claim are repeated $n-2$

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times;

     (vi) all samples are submitted to cleavage and deprotection.

21. A process for synthesizing a sub-kit bound to unlabeled support comprising all vicinal type second order sub-libraries of a parent peptide library, in which a sample of solid support, carrying blocked primary amino groups attached through a spacer, is consecutively submitted to deprotection, operations (i) and (viii) of claim 10, then the samples are treated according operations (i) to (v) of claim 20, and finally all samples are submitted to deprotection.

22. A process for synthesizing a sub-kit comprising all vicinal type second order sub-libraries of a parent peptide library of soluble peptides, carrying color or fluorescent labels at their C-terminus, assigned to the amino acid residue occupying the lower coupling position of the two vicinal non-varied positions, in which the process is started with p equal samples of solid support comprising N-protected O-aminoacyl groups wherein the aminoacyl group is a color or fluorescent marker, differing in color or fluorescence of the label of the support;

     (i) first, operation (xv) of claim 10 is executed;

     (ii) samples A (if any remaining) are submitted to operation (v) of claim 10, then the resultant A samples are used in the next cycle of operations;

     (iii) samples B are consecutively submitted to operations (ii), (iii), (i) and (ii) of claim 10, then the resultant samples are submitted to $n-r$ consecutive cycles of operation (v) of claim 10 then the samples are taken apart;

     (iv) cycle of operations (i) to (iii) of this claim is repeated ($n-2$) times;
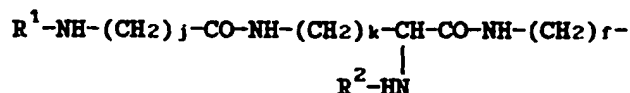
     (v) all samples are submitted to cleavage and deprotection.

23. A process for synthesizing a sub-kit comprising all vicinal type second order sub-libraries of a normal parent library of peptides bound to color or fluorescent supports, when the color or fluorescence or the size or the specific gravity of the support is assigned to the amino acid residue occupying the lower coupling position of the two vicinal non-varied positions, and in which the process is started with p equal

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samples of polymer and glass supports comprising free or protected primary
amino groups, wherein the beads of different size are colored or
fluorescent, differing in at least one of the mentioned characteristics of
the support, then the samples are submitted to operations (i) to (iv) of
claim 22, finally all samples are submitted to deprotection.

24. A process for synthesizing a sub-kit comprising all vicinal type
second order sub-libraries of a normal parent library of peptides bound to
color or fluorescent supports, when the color or fluorescence of the
support is assigned to the amino acid residue occupying the lower coupling
position of the two vicinal non-varied positions, and in which the process
is started with a single deprotected sample of solid support comprising
acylaminoalkyl groups corresponding to the following general formula where
R^1 and R^2 are protecting groups, j, k, and r are integers 1 to 6

$$R^1\text{-NH-(CH}_2)_j\text{-CO-NH-(CH}_2)_k\text{-CH-CO-NH-(CH}_2)_r\text{-}$$
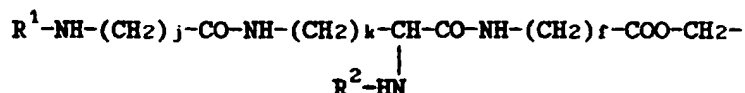$$R^2\text{-HN}$$

and the color or fluorescent labels are attached to the support in the
course of the peptide synthesis;

        (i) the sample is submitted to operations (i) and (ii) of claim
        10;

        (ii) the samples are submitted to operation (xiii) of claim 10;

        (iii) samples A (if any remaining) are consecutively submitted
to operations (iii), (i), (ii) of claim 10 then the resultant A
samples are used in the next cycle of operations;

        (iv) samples B are submitted to operation (x) of claim 10, then
mixed and submitted to operations (i) and (ii) of claim 10 then the
resultant samples are submitted to $n-r$ consecutive cycles of
operation (v) of claim 10 then the samples are taken apart;

        (v) cycle of operations (ii) to (iv) of this claim is repeated
$n-2$ times;

        (vi) all samples are submitted to deprotection.

25. A process for synthesizing a sub-kit comprising all vicinal type
second order sub-libraries of a normal parent library of peptides bound to
color or fluorescent supports, when the color or fluorescence of the

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support is assigned to the amino acid residue occupying the lower coupling position of the two vicinal non-varied positions, and in which the process is started with a single deprotected sample of polymer or glass supports comprising O-acylated hydroxymethyl groups corresponding to the following general formula where R^1 and R^2 are protecting groups, j, k, and r are integers 1 to 6

$$R^1\text{-NH-(CH}_2)_j\text{-CO-NH-(CH}_2)_k\text{-CH-CO-NH-(CH}_2)_r\text{-COO-CH}_2\text{-}$$
$$\underset{R^2\text{-HN}}{|}$$

and the color or fluorescent labels are introduced in the course of the peptide synthesis;

(i) the sample is submitted to operations (i) to (v) of claim 24;

(ii) all samples are submitted to deprotection and cleavage.

26. A method for determination of the amino acid sequences of bioactive peptides ("domino strategy") using kits according to claims 1 to 9 whereby
the use of unlabeled sub-kits comprises three consecutive steps;

(i) determination of the amino acid occurrence list by executing the screening experiments with all components of the amino acid tester sub-kit;

(ii) determination of the occurrence library by carrying out screening experiments with selected components of the first sub-kit;

(iii) determination of the amino acid sequences by the domino like use in screening of selected components of the second sub-kit(s) of the parent peptide library or by synthesizing the second sub-kit of the occurrence library and doing screening tests with its components;

while when using labelled kits the experiments described in (i) can be omitted, those of (ii) are executed with all components of the first sub-kit, and (iii) is applied unchanged.

27. Polymer and glass supports comprising free or protected primary amino groups, wherein the beads are colored or fluorescent.

28. Polymer and glass support comprising acylaminoalkyl groups corresponding to the following general formula where R^1 and R^2 are protecting groups, j, k, and r are integers 1 to 6.

$$R^1\text{-NH-(CH}_2)_j\text{-CO-NH-(CH}_2)_k\text{-CH-CO-NH-(CH}_2)_r\text{-}$$
$$\underset{R^2\text{-HN}}{|}$$
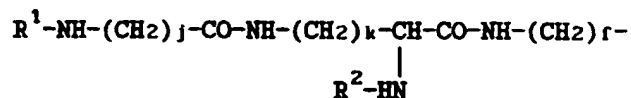
29. Polymer or glass supports comprising N-protected O-aminoacyl groups wherein the aminoacyl group is a color or fluorescent marker.

30. Polymer or glass supports comprising O-acylated hydroxymethyl groups corresponding to the following general formula where R^1 and R^2 are protecting groups, j, k, and r are integers 1 to 6.

$$R^1\text{-NH-(CH}_2)_j\text{-CO-NH-(CH}_2)_k\text{-CH-CO-NH-(CH}_2)_r\text{-COO-CH}_2\text{-}$$
$$\underset{R^2\text{-HN}}{|}$$
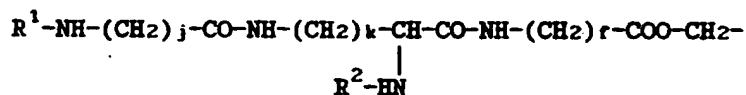
31. Polymer or glass supports according to any of claims 27 and 29 derived by the use of 4-[4-[N-Ethyl-N-[3-[tert-butyloxycarbonyl]amino-propyl]amino]phenylazo]benzoic acid (EPAP) as color marker.

32.   4-[4-[N-Ethyl-N-[3-[tert-butyloxy-carbonyl]aminopropyl]amino]-phenylazo]benzoic acid (EPAP).